

Noncompetitive enzyme immunoassay for carcinoembryonic antigen by flow injection chemiluminescence

Jiehua Lin^a, Feng Yan^b, Huangxian Ju^{a,*}

^aDepartment of Chemistry, Institute of Analytical Science, State Key Laboratory of Coordination Chemistry, Nanjing University, Nanjing 210093, PR China

^bJiangsu Institute of Cancer Prevention and Cure, Nanjing 210009, PR China

Received 8 September 2003; received in revised form 17 November 2003; accepted 18 November 2003

Abstract

Background: Recently, many automated immunoassay analyzers have been developed for carcinoembryonic antigen (CEA) to overcome the shortcomings in traditional immunoassay methods that are time-consuming and labor-intensive. Flow injection immunoassay (FIIA) has been increasingly applied to laboratory medicine due to its ease in automation, rapid speed and reproducible results. It is important to develop a FIIA method for CEA determination. **Methods:** Based on a noncompetitive immunoassay format, a CEA-immobilized immunoaffinity column inserted in the flow system was used to trap the unbound horseradish peroxidase (HRP)-labeled antibody after an off-line incubation of CEA and HRP-labeled anti-CEA. The trapped enzyme conjugate was detected by injecting substrates to produce an enhanced chemiluminescence (CL). **Results:** The linear range for CEA was 1.0–25 ng/ml with a correlation coefficient of 0.997 and a detection limit of 0.5 ng/ml. The sampling and chemiluminescence detection time for one sample was 5 min after a preincubation procedure of 25 min. Twenty five human serum samples detected by this method were in good agreement with the results obtained by immunoradiometric assay (IRMA). **Conclusions:** This method could be used for rapid analysis of CEA and potentially other antigens.

© 2004 Elsevier B.V. All rights reserved.

Keywords: Flow injection analysis; Chemiluminescence; Noncompetitive enzyme immunoassay; Immunoaffinity; Carcinoembryonic antigen (CEA); Horseradish peroxidase

1. Introduction

In the management of cancer patients, tumor-associated antigens in serum have been used in noninva-

sive tests for relapse detection [1]. Carcinoembryonic antigen (CEA), an important tumor-associated antigen, is an acidic glycoprotein with a molecular weight of about 200 kDa and has been expressed in many malignancies [2,3]. The concentration of serum CEA is related to colon cancer [2–4], lung cancer [5,6], ovarian carcinoma [7] and breast cancer [8–10]. The detection of serum CEA level plays an important role in monitoring patients after therapy.

The traditional assay for serum CEA is radioimmunoassay and enzyme immunoassay (EIA), which

Abbreviations: CEA, Carcinoembryonic antigen; CL, chemiluminescence; CV, variation coefficient; EIA, enzyme immunoassay; FIIA, Flow injection immunoassay; H₂O₂, hydrogen peroxide; HRP, horseradish peroxidase; IRMA, Immunoradiometric assay; PBS, phosphate buffer solution; PIP, *p*-iodophenol.

* Corresponding author. Tel./fax: +86-25-3593593.

E-mail address: hxju@nju.edu.cn (H. Ju).

are based on the specificity and selectivity of antibody–antigen reaction [11,12]. There is an ongoing effort to eliminate radioactive materials in immunoassays. Conventional EIA has the disadvantages of being time-consuming, have poor precision and are difficult to automate. Recently, a fully automated, random-access, Walk-way immunoassay analyzers have been used in clinical practice because of its efficient use of labor, minimal sample and reagent manipulation, easy automation and flexibility [13,14]. Here, we combine flow injection analysis (FIA), introduced in 1975 [15], to immunoassay and establish the flow injection immunoassay (FIIA), for the detection of tumor markers. Compared to the existing automated method, the FIIA requires simpler and cheaper instrumentation and has better reproducibility. This technique has been applied to many fields such as food, pharmaceutical, environmental and clinical assays [16–22].

The sensitivity of EIA depends on both the detection method and the sensitivity of the enzyme labeled on immunoreagent [23]. The enzymes used widely as labels include horseradish peroxidase (HRP) and alkaline phosphatase [24], which can be detected by photometry, fluorescence or chemiluminescence (CL) techniques [17]. The combination of CL with FIA is useful because CL is relatively simple, has very low detection limits and wide dynamic range [25]. HRP is commonly used in CL detection by catalyzing the luminol oxidation of hydrogen peroxide (H_2O_2). To detect HRP at a very low level, several compounds such as phenol derivatives [26,27], carbon dioxide [28], fluorescein [29] and phenylboronic acid [30] have been used to enhance the emission intensity of luminol chemiluminescent system. Although *p*-iodophenol (PIP) has been used for immunoassay for rabbit IgG [16], study of real-time antigen–antibody interaction [31] and dichlorprop methyl ester [32] and DDT [33] by the enhanced chemiluminescent methods, it has not been used in the determination of tumor marker. The noncompetitive FIIA was proposed in 1993 [23] to detect a hapten by measuring fluorometrically the HRP-labeled antibody–hapten complex, which was separated from the free HRP-labeled antibody by binding the latter to the affinity column. It overcomes the limitations presented in competitive assay [34], is faster than a conventional EIA and can be fully automated [23,35].

2. Materials and methods

2.1. Materials and reagents

Horseradish peroxidase (HRP, Sigma), CEA standard solutions (Wallac Oy, Turku, Finland), HRP-labeled anti-CEA solution (Everlong, CA, USA), CNBr-activated Sepharose™ 6MB (Amersham Pharmacia Biotech, Sweden), luminol (Shanxi Normal University, China), *p*-iodophenol (PIP, Weihai New-era Chemical, China) and H_2O_2 (Shanghai Chemical Plant, China) were used. All other reagents were of analytical-reagent grade and used without further purification. Luminol, 0.01 mol/l, and 0.01 mol/l PIP stock solutions were kept in the dark and diluted using 0.1 mol/l pH 8.5 Tris–HCl buffer solution prior to use. H_2O_2 working solution was prepared by appropriate dilution of 30% solution with 0.1 mol/l pH 8.5 Tris–HCl buffer solution daily.

2.2. Preparation of immunoaffinity columns

The following buffers were used to prepare antigen-modified sepharose for preparation of immunoaffinity column: coupling buffer (0.1 mol/l NaHCO_3 /0.5 mol/l NaCl, pH 8.0), washing buffers (0.1 mol/l CH_3COONa – CH_3COOH /0.5 mol/l NaCl, pH 4.5 and 0.1 mol/l Tris–HCl/0.5 mol/l NaCl, pH 8.0) and blocking buffer (0.1 mol/l NaHCO_3 /0.2 mol/l glycine, pH 8.5). The preparation of immunoaffinity column was performed according to Amersham Pharmacia Biotech. Freeze dried powder of CNBr-activated Sepharose™ 6 MB (100 mg) was suspended in 1 mmol/l HCl. The swollen sepharose was washed with 1 mmol/l HCl on a sintered glass filter for 15 min and washed with the coupling buffer and then added to 300 μl CEA of 500 ng/ml to the sepharose gel. The mixture was incubated for 24 h at 4 °C and then mixed with the blocking buffer for another 24 h at 4 °C. Finally, it was washed with pH 4.5 and 8.0 washing solutions for three cycles and filled to glassy tube (1.6 mm inner diameter and 10 cm length).

2.3. Instrumentation

The immunoaffinity column was connected to a flow system illustrated in Fig. 1. Two peristaltic

pumps of Luminescence Analyzer (IFFM-D, Remex Electronic Instrument, Xi'an, China) were used to deliver flow streams. Polytrafluoroethylene tubing (0.8 mm i.d.) was used to connect all components in the flow system. A multiway valve was used not only to shift the directions of the fluids, but also used as a chemiluminescent substrates injection valve. A colorless spiral glass tube (2.0 mm inner diameter and 10 cm length) was used as the flow cell, which was connected to the immunoaffinity column with a distance as short as possible. The CL emission was detected by the photo-multiplier tube (PMT) placed near the flow cell and recorded.

2.4. Noncompetitive flow injection immunoassay

A diagram of the noncompetitive enzyme immunoassay was shown in Fig. 2. Phosphate buffer solution (PBS), 0.1 mol/l, pH 7.0 was used as the carrier. A 50- μ l sample of diluted HRP-labeled anti-CEA solution in 0.1 mol/l pH 7.0 PBS was mixed with 50 μ l CEA standard solution at room temperature for 25 min. One hundred microliters of the immunomixture was taken rapidly to the front end of the immunoaffinity column, then carried by PBS through the column at a flow rate of 0.1 ml/min. At the same time, luminol, PIP and H₂O₂ were mixed. After the unbound enzyme conjugate was trapped by CEA modified gel, 100 μ l mixture of these CL substrates was injected by turning the valve and carried to the column. The CL signal was detected and recorded. The modified gel was regenerated by 0.1 mol/l pH 2.2 glycine-HCl and then equilibrated by 0.1 mol/l pH 7.0 PBS. The total detection time

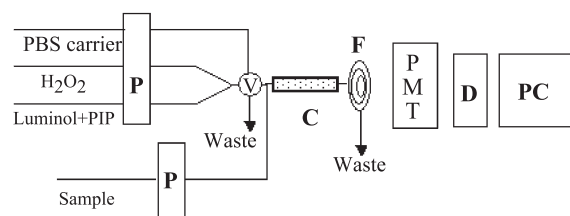


Fig. 1. Schematic diagram of the chemiluminescent noncompetitive FIIA system: (P) peristaltic pump, (V) eight-way valve, (C) immunoaffinity column, (PMT) photo-multiplier, (D) detector and (PC) computer.

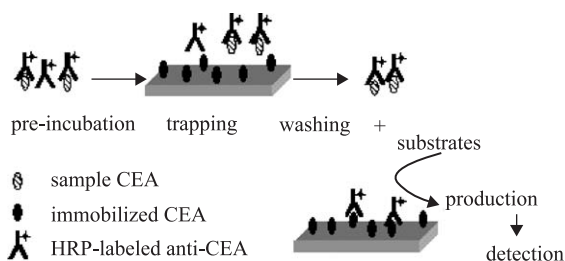


Fig. 2. Schematic diagram of noncompetitive enzyme immunoassay procedure for CEA.

including sampling and the regeneration of 2 min was about 5 min.

3. Results and discussion

3.1. Optimization of CL detection system

FIIA performance depends on both assay procedure and the characteristics of substrates. CL emission intensity is sensitive to a variety of environmental factors such as temperature, solvent, ionic strength, solution pH and other species present in the system. We examined the effects of the concentrations of luminol and H₂O₂, CL detection buffer solution and flow rate on CL intensity in presence of HRP-labeled anti-CEA with a dilution of 1:20. The CL intensity reached the maximum value at the H₂O₂ and luminol concentrations of 1.0 and 0.5 mmol/l when the concentrations of luminol and H₂O₂ were set at 0.5 and 1.0 mmol/l, respectively. The CL emission in the Tris-HCl buffer was more stable than in other buffers such as PBS, Na₂CO₃-NaHCO₃. Under optimal conditions, the CL emission of luminol-H₂O₂ system was enhanced upon addition of PIP, which accorded with the conclusion reported in Ref. [16]. As seen in Fig. 3, with an increasing PIP concentration, the CL emission increased and reached a maximum value at 0.4 mmol/l. Thus, 0.1 mol/l pH 8.5 Tris-HCl buffer solution containing 1.0 mmol/l H₂O₂, 0.5 mmol/l luminol and 0.4 mmol/l PIP was selected as the CL detection solution. Considering the stability and sensitivity of CL emission, the flow rates of luminol-PIP mixture and H₂O₂ were selected at 0.9 ml/min, respectively. The total flow rate of substrates through the column to the flow cell was 1.8 ml/min.

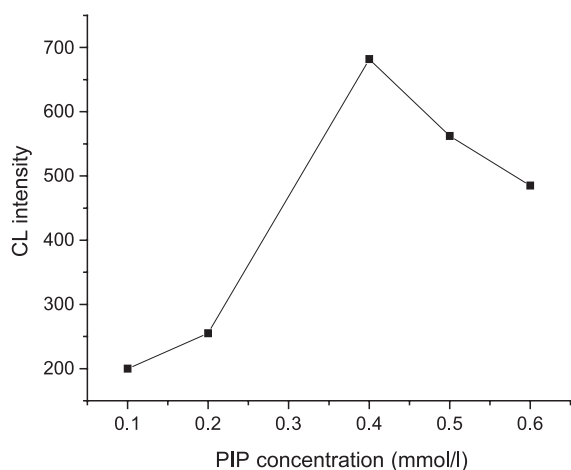


Fig. 3. Effect of PIP concentration on CL intensity of 1.0 mmol/l H_2O_2 and 0.5 mmol/l luminol in the presence of HRP-labeled anti-CEA with a dilution of 1:20.

3.2. Optimization of immunoassay procedure

The performance evaluation of immunoassay system included the optimization of the working concentration of HRP-labeled anti-CEA, preincubation time for the immunoreaction between analyte CEA and enzyme conjugate and the residence time of the reaction mixture in the immunoaffinity column.

The working concentration of HRP-labeled anti-CEA was selected to give a compromise with a good sensitivity and a wide assay range. The optimal dilution of HRP-labeled anti-CEA solution was established by injecting different diluted enzyme conjugate solutions into the column. The trapped HRP-labeled antibody enhanced the chemiluminescent reaction when the chemiluminescent substrates flowed through the column. The plot of CL intensity vs. the dilution of the enzyme conjugate solution was shown in Fig. 4. The CL signal trended to a plateau at a dilution of 1:20, which meant that all of the specific sites of immobilized CEA were occupied. Thus, 1:20 dilution of HRP-labeled anti-CEA was chosen as the working solution in this work.

A sample containing 5.0 ng/ml CEA was incubated with a 1:20 dilution of enzyme conjugate at room temperature for different time intervals, and then the solution was injected into the flow system to obtain an optimal preincubation time (Fig. 5). With an increas-

ing preincubation time, the CL signal decreased and trended to a minimum value after a preincubation time of 25 min, indicating a maximum combination of CEA with enzyme conjugate. A preincubation time of 25 min was chosen for CEA detection.

The residence time of the immunomixture in the immunoaffinity column depended on both the column dimension and the flow rate of the mixture through the column. The flow rate decided the binding efficiency of the free enzyme conjugate to the immobilized CEA on the gel surface. At a high flow rate, the free enzyme conjugate was partially removed from the preincubation mixture due to a short residence time. The complete capture of the free enzyme conjugate needed a long time, which was disadvantageous to practical application of this method. Herein, the column dimension was fixed at 1.6 mm inner diameter and 10 cm length. Fig. 6 shows the effect of the flow rate on the trapping efficiency of the immunoaffinity column to the free enzyme conjugate. The output signal increased with a decreasing flow rate, indicating the flow rate of the preincubation mixture through the column was an important factor for the efficient trapping of the free enzyme conjugate. At a flow rate of 0.1 ml/min, at which the time of the preincubation mixture through the column was about 2 min, the capture of the free enzyme conjugate could reach 70% of that at a static state for 30 min. Considering the whole analytical time

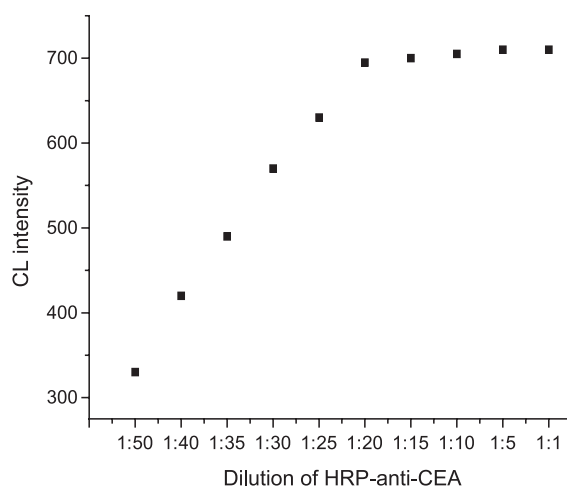


Fig. 4. Effect of HRP-labeled anti-CEA dilution on CL intensity of 1.0 mmol/l H_2O_2 , 0.5 mmol/l luminol and 0.4 mmol/l PIP.

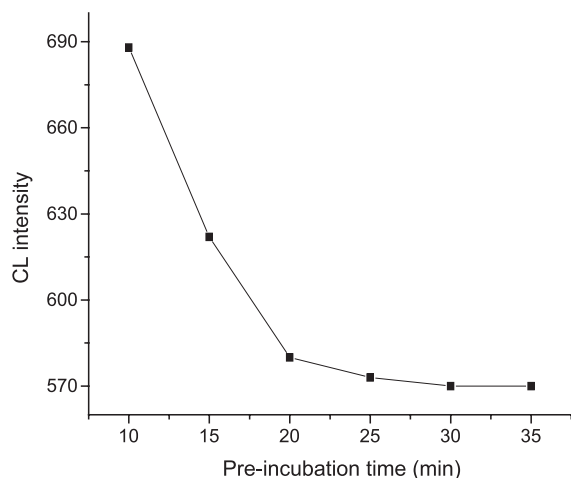


Fig. 5. Dependence of CL intensity on preincubation time of 5.0 ng/ml CEA with enzyme conjugate of 1:20 dilution at room temperature.

and the practical application of this method in serum CEA detection in batch, 0.1 ml/min was selected as the flow rate of the preincubation mixture or sampling rate.

3.3. Noncompetitive flow injection immunoassay for CEA

Under optimal conditions, typical CL signals obtained with the noncompetitive immunoassay for

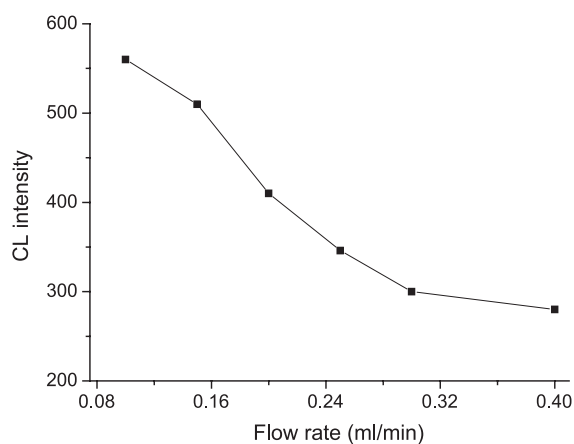


Fig. 6. Effect of flow rate of preincubation mixture after a preincubation time of 25 min through the immunoaffinity column on the trapping efficiency of the free enzyme conjugate. Other conditions are the same as in Fig. 5.

CEA were shown in Fig. 7. The black signal was measured when 0.1 mol/l pH 8.5 Tris–HCl buffer solution containing 1.0 mmol/l H_2O_2 , 0.5 mmol/l luminol and 0.4 mmol/l PIP flowed through the column at a total flow rate of 1.8 ml/min without the trapping of HRP-labeled anti-CEA. With the increasing CEA concentration, the CL signal decreased and trended to the black value at the CEA concentrations >25 ng/ml, indicating most of HRP-labeled anti-CEA in the preincubation solution bound with CEA; thus, little HRP-labeled anti-CEA was trapped in the column. The plot of relative CL signal (the difference between the CL signal in presence of trapped HRP-labeled anti-CEA and the black value) vs. the CEA concentration showed a linear decrease from 1.0 to 25 ng/ml with a correlation coefficient of 0.997 (inset in Fig. 7). The precision of CEA at 5 ng/ml ($n=5$) was 2.5%. The assay time including preincubation step was 30 min for per sample. The detection limit, taken as the concentration equivalent to a 10% decrease in signal [36–38], was 0.5 ng/ml, well within the requirement for clinical analysis. When CEA concentration was >25 ng/ml, the detection needed a dilution of sample or higher concentration of HRP-labeled anti-CEA used in the preincubation step. The latter needed to obtain a new calibration curve for CEA detection.

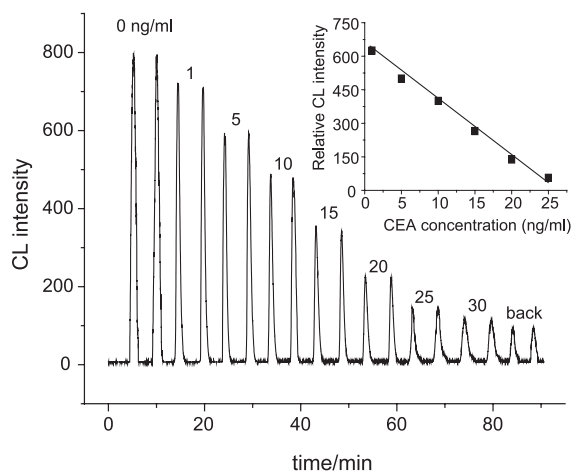


Fig. 7. FIA diagram from noncompetitive immunoassay of CEA at different concentrations at a sampling interval of 5 min. Black peaks were generated from the CL reaction between substrates without sampling steps. Inset: calibration curve for CEA.

3.4. Accuracy and clinical application

The accuracy of the CEA determination was examined by comparing the results obtained with this method and immunoradiometric assays (IRMAs). The mean CEA concentrations in 25 sera samples were determined with IRMA and this proposed method. The relative deviations of these results between the two methods were in $-8.80\sim 10.0\%$.

3.5. Stability of the immunoaffinity column

The stability of the immunoaffinity column was assessed by intra- and inter-assay variation coefficients (CVs). The intra-assay CV was the difference among three determinations of one sample on the same column after the column was regenerated. The inter-assay CV was the difference among the measurements of the same sample on three different columns. The intra- and inter-assay CVs obtained at CEA concentration of 5.0 ng/ml were 1.42% and 8.10%, respectively. The low value of intra-assay CV indicated the column could be regenerated and used repeatedly. After the column was regenerated for 30 times, the analytical performances did not show significant decrease.

4. Conclusions

A new simple enzyme immunoassay for CEA by combining noncompetitive flow injection with enhanced CL detection is described. The free enzyme conjugate is separated from the preincubation mixture with an immunoaffinity column and detected by injecting the chemiluminescent substrates into the column. In comparison with the conventional EIA approaches, this proposed method is simpler, sensitive (0.5 ng/ml) and rapid (5 min). This method could be further developed for other antigens requiring fast turnaround times, such as troponin or CK-MB.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 20275017,

90206037), the Specialized Research Funds for the Excellent Young Teachers from Chinese Ministry of Education, the Science Foundation of Jiangsu (No. BS2001063) and the Key Project of Cancer Institute of Jiangsu Province.

References

- [1] Magdelenat H. Tumour markers in oncology: past, present and future. *J Immunol Methods* 1992;150:133–43.
- [2] Schlageter MH, Larghero J, Cassinat B, Toubert ME, Borscheck C, Rain JD. Serum carcinoembryonic antigen, cancer antigen 125, cancer antigen 15-3, squamous cell carcinoma, and tumor-associated trypsin inhibitor concentrations during healthy pregnancy. *Clin Chem* 1998;44:1995–8.
- [3] Gold P, Freedman SO. Demonstration of tumor-specific antigens in human colonic carcinomata by immunological tolerance and absorption techniques. *J Exp Med* 1965;121:439–62.
- [4] Duffy MJ, van Dalen A, Haglund C, Hansson L, Klapdor R, Lamerz R, et al. Clinical utility of biochemical markers in colorectal cancer: European Group on Tumour Markers (EGTM) guidelines. *Eur J Cancer* 2003;39:718–27.
- [5] Kleisbauer JP, Castelnau O, Thomas P, Ramirez J, Lanteaume A, Roux F. Prognostic value of carcinoembryonic antigen in lung carcinoma before treatment. *Lung Cancer* 1996;15:148.
- [6] Hernández L, Espasa A, Fernández C, Candela A, Martín C, Romero S. CEA and CA 549 in serum and pleural fluid of patients with pleural effusion. *Lung Cancer* 2002;36:83–9.
- [7] Engelen MJA, de Bruijn HWA, Hollema H, ten Hoor KA, Willemse PHB, Aalders JG, et al. Serum CA 125, carcinoembryonic antigen, and CA 19-9 as tumor markers in borderline ovarian tumors. *Gynecol Oncol* 2000;78:16–20.
- [8] Sahin B, Paydak V, Paydas S. Hepatosteatosis and alterations of CA15-3 and CEA in patients with breast cancer receiving tamoxifen. *Eur J Cancer* 1996;32:s24.
- [9] Bremer K, Micus S, Bremer G. CEA, CA 15-3 and MCA: comparative clinical relevance in breast cancer. *Eur J Cancer* 1995;31:s262.
- [10] Jezeršek B, Cervek J, Rudolf Z, Novakovic S. Clinical evaluation of potential usefulness of CEA, CA 15-3, and MCA in follow-up of breast cancer patients. *Cancer Lett* 1996;110:137–44.
- [11] Alumanda MDR, Minoru K. Trapping method of antibodies on surfaces of polymerizing discs for enzyme immunoassay. *Anal Chim Acta* 1995;312:85–94.
- [12] Eckert K, Fuhrmann-Selter T, Maurer HR, Büttner P. A colorimetric immunoassay for the detection of E-cadherin and carcinoembryonic antigen (CEA) expression on human colonic carcinoma cell lines in vitro. *Cancer Lett* 1996;105:1–4.
- [13] Hendriks HA, Kortlandt W, Verweij WM. Standardized comparison of processing capacity and efficiency of five new-generation immunoassay analyzers. *Clin Chem* 2000;46:105–11.
- [14] Rymer JC, Sabatier R, Daver A, Bourleaud J, Assicot M, Bremond J, et al. A new approach for clinical biological assay

- comparison and standardization: application of PCA to a Multicenter Study of Twenty-One CEA Immunoassay Kits. *Clin Chem* 1999;45:869–81.
- [15] Ruzicka J, Hansen E. Flow injection analysis: Part I. A new concept of fast continuous flow analysis. *Anal Chim Acta* 1975;78:145–57.
- [16] Liu HJ, Yu JC, Bindra DS, Givens RS, Wilson GS. Flow injection solid-phase chemiluminescent immunoassay using a membrane-based reactor. *Anal Chem* 1991;63:666–9.
- [17] Luo JX, Yang XC. Flow injection chemiluminescent immunoassay with *para*-phenylphenol and sodium tetraphenylborate as synergistic enhancers. *Anal Chim Acta* 2003;485:57–62.
- [18] Locascio-Brown L, Plant AL, Horváth V, Durst RA. Liposome flow injection immunoassay: implication for sensitivity, dynamic range and antibody regeneration. *Anal Chem* 1990;62:2587–93.
- [19] Nandakumar R, Nandakumar MP, Mattiasson B. Quantification of nisin in flow-injection immunoassay systems. *Biosens Bioelectron* 2000;15:241–7.
- [20] Berezcki A, Horváth V. Novel type of flow injection immunoassay for the determination of phenytoin in serum. *Anal Chim Acta* 1999;391:9–17.
- [21] Bjarnason B, Bousios N, Eremin S, Johansson G. Flow injection enzyme immunoassay of atrazine herbicide in water. *Anal Chim Acta* 1997;347:111–20.
- [22] Burestedt E, Kjellström S, Emnéus J, Marko-Varga G. Development of an offline noncompetitive flow immunoassay for the determination of interleukin-8 in cell samples. *Anal Biochem* 2000;279:46–54.
- [23] Gunaratna PC, Wilson GS. Noncompetitive flow injection immunoassay for a hapten, α -(difluoromethyl) ornithine. *Anal Chem* 1993;65:1152–7.
- [24] Zhu QZ, Liu FH, Li DH, Xu JG, Su WJ, Huang JW. A novel polymer-mimetic enzyme immunoassay system based on thermal phase separating technique. *Anal Chim Acta* 1998;375:177–85.
- [25] Baeyens WRG, Schulman SG, Calokerinos AC, Zhao Y, Campaña AMG, Nakashima K, et al. Chemiluminescence-based detection: principles and analytical applications in flowing streams and in immunoassays. *J Pharm Biomed Anal* 1998;17:941–53.
- [26] Dodeigne C, Thunus L, Lejeune R. Chemiluminescence as diagnostic tool: a review. *Talanta* 2000;51:415–39.
- [27] Thorpe GHG, Kricka LJ, Gillespie E, Moseley S, Amess R, Baggett N, et al. Enhancement of the horseradish peroxidase catalyzed chemiluminescent oxidation of cyclic diacyl hydrazides by 6-hydroxy benzothiazoles. *Anal Biochem* 1985;145:96–100.
- [28] Zhang HL, Horacio AM. Carbon dioxide-enhanced luminol chemiluminescence in the absence of added oxidant. *Analyst* 1996;121:211–8.
- [29] Navas Diaz A, Gonzalez Garcia JA, Lovillo J. Enhancer effect of fluorescein on the luminol-H₂O₂-HRP chemiluminescence: energy transfer process. *J Biolumin Chemilumin* 1997;12:199–205.
- [30] Kricka LJ, Ji XY. Synergistic enhancement of the horseradish peroxidase-catalyzed oxidation of luminol by 4-substituted phenylboronic acids. *Talanta* 1997;44:1073–9.
- [31] Osipov AP, Zaitseva NV, Egorov AM. Chemiluminescent immunoenzyme biosensor with a thin-layer flow-through cell. Application for study of a real-time bimolecular antigen–antibody interaction. *Biosens Bioelectron* 1996;11:881–7.
- [32] Diaz AN, Sánchez FG, Lovillo J, García JAG. Enhanced chemiluminescence kinetic ELISA of dichlorprop methyl ester. *Anal Chim Acta* 1996;321:219–24.
- [33] Botchkareva AE, Fini F, Eremin S, Mercader JV, Montoya A, Girotti S. Development of a heterogeneous chemiluminescent flow immunoassay for DDT and related compounds. *Anal Chim Acta* 2002;453:43–52.
- [34] Jackson TM, Ekins RP. Theoretical limitations on immunoassay sensitivity: current practice and potential advantages of fluorescent europium (3+) chelates as non-radioisotopic tracers. *J Immunol methods* 1986;87:13–20.
- [35] Lövgren U, Kronkvist K, Bäckström B, Edholm LE, Johansson G. Design of non-competitive flow injection enzyme immunoassays for determination of haptens: application to digoxigenin. *J Immunol Methods* 1997;200:159–68.
- [36] Gascón J, Durand G, Barcelo D. Pilot survey for atrazine and total chlortrazines in estuarine waters using magnetic particle-based immunoassay and gas chromatography-nitrogen/phosphorous detection. *Environ Sci Technol* 1995;29:1551–6.
- [37] Keay RW, McNeil CJ. Separation-free electrochemical immunosensors for rapid determination of atrazine. *Biosens Bioelectron* 1998;13:963–70.
- [38] Dai Z, Yan F, Chen J, Ju HX. Reagentless amperometric immunosensors based on direct electrochemistry of horseradish peroxidase for rapid determination of carcinoma antigen-125. *Anal Chem* 2003;75:5429–34.